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A Novel Protocol to Separate Nitrite and Nitrare for their Sequential Determination in Soil, Excreta and Leafy Vegetables by Griess Reaction

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Abstract: A novel procedure has been standardized to enable sequential and selective determinations of nitrite and nitrate in the same sample by Griess reaction. Nitrite is determined by coupling of diazotized sulfanilamide and N-1-naphthyl-ethylenediamine (NEDA) to form a chromophore at room temperature. The chromophore and the residual NEDA are removed from the solution by addition of suspension of activated charcoal (2.5 % in ca. 3.6 M acetate buffer, pH 6.6) @ 0.25 mL per mL solution. The suspension mixture is centrifuged and filtered after 15 minutes. The filtrate is assayed for nitrate by acid reduction and diazotization at elevated temperature, and coupling to NEDA at room temperature. High acetate ensures 101 ± 2 % recovery of nitrate compared to 81 ± 1 % recovery in absence of acetate. The technique is based on high efficiency of activated charcoal to adsorb sulfanilamide, diazotized sulfanilamide and azo-dye. The protocol has removed at least up to125 µg nitrite N and up to at least 1000 µg free NEDA from each sample. The applicability of the protocol has been tested on selected samples: soil, sheep excreta and leafy vegetables i.e. kohlrabi and local kale.
Keywords: Activated charcoal, Separation, Nitrite, Nitrate, Azo-dye.

INTRODUCTION

Nitrate and nitrite are established environmental pollutants and toxicants. Nitrate is more stable anion than its toxic metabolite nitrite and is dominant in environmental and biological samples**.** The Griess reaction is highly specific for nitrite. The reaction has been employed to determine nitrate following its chemical or enzymatic reduction to nitrite using a variety of versions[1-5]. A major technical limitation in the techniques is that the nitrate is measured indirectly with measurements made twice, before and after reduction, and the nitrate determined as a difference of NO_x and NO_2[2]. This approach has serious limitations while determining nitrate in presence of nitrite[4]. Differential abilities of nitrite and nitrate to engage in diazotization of sulfanilamide under different experimental conditions have provided a rationale for their sequential analysis in test samples[5]. Urea pretreatment has enabled direct determination of nitrate in presence of high nitrite[4, 5]. A more simplified alternative procedure has been mooted to enable sequential determinations of nitrite and nitrate in the same sample, without using urea, based on the following observations: (i) activated charcoal is capable of removing sulfanilamide quite efficiently[6], (ii) high electrolyte concentration, particularly of acetate, has prevented adsorption of nitrate anion to activated charcoal[7], and (iii) during the course of preliminary investigations, activated charcoal has been found to remove diazotized sulfanilamide as well

as the chromophore formed by diazotized sulfanilamide and coupling agent NEDA. These considerations motivated to standardize the procedure with following objectives: (i) to determine nitrite at room temperature under mild acid conditions without any interference from simultaneously present nitrate[5], (ii) to remove the chromophore and any unreacted NEDA by adsorption to activated charcoal in presence of high acetate concentration without adsorbing nitrate[7], and (iii) to determine nitrate in the filtrate by acid reduction method[5]. The optimized technique has been employed to determine nitrite and nitrate in sheep fecal matter, composite soil samples, and in homogenates of two commonly used leafy vegetables.

MATERIALS AND METHODS

The experiments were carried out at an ambient temperature of 21.4 ± 0.7 ^{0}C. The chemicals used were of analytical grade purity, and included N-1-(naphthyl) ethylenediamine dihydrochloride (NEDA), sodium nitrite, sulfanilamide (Loba Chemie (India) Ltd., Mumbai), sodium hydroxide (E.Merck (India) Ltd, Mumbai), hydrochloric acid (Qualikems Fine Chemicals (India), Ltd, New Delhi), glacial acetic acid, potassium nitrate (S.D.Fine-Chem (India) Ltd, Mumbai) and activated charcoal, magnesium chloride hexahydrate (Glaxo Laboratories (India) Ltd, Mumbai) .Double-distilled water was used for making solutions, dilutions and for final washings of glassware.

Reagents

Nitrite solution: Stock 50 ppm as NO2-N in water. Appropriate dilutions made in water

Nitrate solution: Stock 100 ppm as NO3 -N in water. Appropriate dilutions made in water.

Sulfanilamide: 0.5 and 1 % (w/v) in 1 % HCl.

NEDA reagent: 0.5 and 1 % (w/v) in 1 % HCl. Appropriate dilutions made in 1 % HCl or 15 % HCl as per experimental requirements.

Acetate buffer: Acetate buffer with approximate strength of 3.6 M sodium acetate was prepared by dissolving sodium hydroxide pellets 12 g in 70 ml water with addition of 20 mL glacial acetic acid. The mixture was cooled to room temperature, and pH adjusted about 6.6 with dilute NaOH (1M) and dilute HCl (10%). Volume was made 100 mL with water.

Purified activated charcoal: Activated charcoal was purified to get rid off residual nitrate and nitrite. The particle size was less than 0.125 mm as it passed wholly through 125 micron mesh (ASTM), and about 10 per cent was retained by 90 micron mesh (ASTM). Approximately 50 g activated charcoal was shaken with 500 ml of 0.1M NaOH, boiled for 15 minutes, filtered over Buchner funnel, washed by percolation using about 500 ml 0.2 N HCl, finally washed with deionized water till effluent failed to show any detectable nitrite and nitrate. The purified charcoal was allowed to drain off its liquid, dried in a glass flask over sand bath on hot plate to a free flowing powder, cooled to room temperature, and stored in a well-stoppered glass container. Charcoal suspensions, 2.5 % (w/v) were prepared both in acetate buffer as well as in distilled water.

Diazo reagent: The reagent contained 1 ppm nitrite nitrogen and 0.1 % sulfanilamide in 15 % HCl. The reagent was prepared by addition of 1 mL of 100 ppm nitrite nitrogen solution, 100 mg of sulfanilamide and 15 mL of HCl to make 100 mL in water.

Samples

The samples tested included composite top-soil (within 5 -15 cm soil depth from the

Faculty premises) powder, dried powder from sheep fecal pellets and freshly collected leafy vegetables including kohlrabi (*Brassica olereceae var caulorapa*, locally called Mounji Haak), and local kale (*Brassica olereceae var Haka*, locally called Haak) The vegetables were washed with deionized water, drained dry, and homogenized to a pasty consistency in an electric blender. Kohlrabi leaves and stems were homogenized and processed separately.

Optimization studies

Recovery of nitrate in presence of charcoal in acetate buffer

A 4 mL sample containing 0.75, 7.5 and 25 µg nitrate nitrogen in 15 % HCl including 1 mg each of sulfanilamide and NEDA was added 1 mL of acetate buffered charcoal suspension. The samples were allowed to stand with intermittent mixing for 10-15 minutes, centrifuged and filtered. Each mL filtrate, representing 0.15, 1.5 and 5 µg nitrate nitrogen, assayed for nitrate with respect to simultaneously run standards in absence of charcoal but containing 1 mL acetate buffer.

In a follow-up experiment, nitrate nitrogen 25 µg in 4 mL samples containing 15 % HCl, 1 mg sulfanilamide and 1 mg NEDA were added separately 1 mL charcoal suspension made in acetate buffer, and in water. The samples were processed as per outlined procedure, and filtrates assayed for nitrate using appropriate standards and blanks made in acetate buffer and in water.

Recovery of nitrate in presence of high nitrite

A 3 mL sample containing 7.5 µg NO_3 –N and 50 µg NO_2 –N was added 0.6 mL HCl, 0.2 mL sulfanilamide, 0.2 mL NEDA and 0.2 mL water. The samples were allowed to develop intense color due to azo dye formation for standing for 30 minutes. Each sample was added 1 mL charcoal suspension, allowed to stand for 10-15 minutes with intermittent shaking, centrifuged and filtered. The filtrates were observed

for any visible coloration, and assayed for nitrite and nitrate. Absorbance values and recovery were measured using regent blank and simultaneously run standard without containing nitrite.

Recovery of nitrate with prolonged adsorption

Samples containing NO_2 –N 0.2, 0.5 and 1.0 µg with, respectively, 5, 10 and 20 µg NO_3 –N in 4 mL volume were added each 0.2 mL sulfanilamide solution, 0.6 mL HCl and then 0.2 mL NEDA reagent (n= 3 each). The color was monitored at 540 nm after 30 minute standing at room temperature. Following analysis, each 4 mL colored sample was added 1 mL of charcoal suspension in acetate buffer. The samples stoppered, mixed well and kept in dark overnight. The samples were centrifuged after about 20 hours standing, and processed for nitrate determination using appropriate standards in acetate buffer, and reagent blanks.

Capacity of charcoal to remove nitrite and unreacted coupling agent

A 4 mL sample containing NO_2 –N 50, 75, 100 and 125 µg in 15 % HCl with 1 mg sulfanilamide and 1 mg NEDA was allowed to develop color to their maximal capacities. The colors ranging from dark violet to almost blackish intensity. The samples were treated with 1 mL charcoal suspension in acetate buffer, and filtrates observed for apparent coloration as well as assayed for any unreacted nitrite. Besides, an improvised method was standardized to enable quantitative determination of NEDA. Samples containing 1, 2, 3, 4 and 5 mg coupling agent, NEDA, in 4 mL 15 % HCl were added each 1 mL charcoal suspension in acetate buffer. The samples were mixed and allowed
standing for 10-15 minutes, then centrifuged and filtered. Each mL filtrate was assayed for coupling agent by adding 1 mL diazo reagent and 1 mL 15 % HCl. The color monitored at 540 nm following 30 minute standing. The effect of varying masses of coupling agent on acid reduction and diazotization of nitrate at elevated temperature was simultaneously conducted.

Sequential determination of nitrite and nitrate in test samples

Extraction

Sheep fecal powder 1 g, composite soil powder 12.5 g , leaf and stem homogenates of kohl-rabi 10 g , and local kale homogenate 10 g each were separately added 15 mL water, and allowed to stand at room temperature for 1 hour with vigorous intermittent shakings.

Each sample was added 5 mL 40 % (w/v) lead acetate solution, allowed to stand about 5 minutes, centrifuged at 6000 rpm for 5 minutes and filtered over Whatman No. 1 filter paper. Each 8 mL filtrate was added 1 mL 5M NaOH, mixed and allowed to stand 2-3 minutes, and added 1 mL 50% magnesium chloride solution, mixed, centrifuged and filtered. Lead acetate filtrates from local kale were additionally added activated charcoal @ 10 mg mL^{-1}, and then processed. This was necessitated to remove an interfering agent (?) that imparted otherwise a greenish-yellow discoloration upon addition of sulfanilamide and HCl, in that order (when the order of addition of reagents was reversed, no such discoloration was observed but nitrite estimates were drastically reduced), and significantly reduced the estimated values of nitrite and nitrate. It appears that in absence of sulfanilamide, nitrous acid formed is engaged by the interfering agent leaving it unavailable for formation of diazonium with sulfanilamide. Standard samples contained NO2 –N 10 µg and nitrate nitrogen 200 µg in 15 mL volume, and were similarly treated like samples. Reagent blanks were also treated like samples.

Nitrite determination

Each 4 mL filtrate from the samples and blank was added 0.2 mL 0.5 % sulfanilamide solution and 0.6 mL HCl, and allowed to diazotize 2-3 minutes followed by addition of 0.2 mL of 0.5 % coupling agent. The samples were monitored at 540 nm following standing for at least 30 minutes. Standard sample filtrates were similarly treated while using 0.5, 1.0, 2.0, 3.0 and 4.0 mL filtrates and volume made 4.0 mL each with reagent blank. Mass of NO_2 –N in samples was determined in terms of simultaneously run standard calibration curve.

Removal of azo-dye and any unreacted coupling agent

Following determination of nitrite, the samples containing the chromophore were treated with charcoal suspension @ 0.25 mL per mL colored mixture. The samples were mixed intermittently and allowed to stand for 10-15 minutes. Thereafter, the samples were centrifuged at 5000-6000 rpm for 5 minutes, and filtered over Whatman No. 1 filter paper. Filtrate was assayed for nitrate.

Determination of nitrate

Each mL filtrate, or appropriately diluted filtrate depending upon NO_3 –N concentration, was added 0.1 mL 1 % sulfanilamide solution and 2 mL concentrated HCl. The samples were incubated in boiling water bath for exactly 3 minutes, cooled by immersion in tap water and added 0.4 mL 0.5 % NEDA reagent. The samples were well-mixed and allowed to stand for about an hour and read at 540 nm against simultaneously run standard calibration curve with appropriate reagent blanks.

Table I. Nitrate recovery following treatment with acetate buffered charcoal

NO_3-N, µg	Absorbance, standard[a]	Charcoal treatment Absorbance[b]	Recovered NO_3-N, µg	Recovery %
0.15	0.026± 0.001	0.027± 0.001	0.15 ± 0.01	102 ± 5
1.5	0.202 ± 0.002	0.204 ±0.002	1.51 ± 0.01	101 ± 1
5.0	0.516 ± 0.009	0.524 ±0.008	5.07 ± 0.08	102 ± 2

[a] r = 0.996, b = 0.0988; [b] r = 0.996, b = 0.1003; P >0.1 The values are mean ± se of six observations each

Table II. Adsorption of coupling agent by acetate buffered charcoal

Coupling agent Total mg	Concentration µg mL^{-1}	Recovery with charcoal treatment Recovered µg mL^{-1}	Mean reduction %
1	200	0	100
2	400	0.14 ± 0.01	99.9
3	600	1.6 ± 0.10	99.7
4	800	4.3 ± 0.10	99.4
5	1000	5.0 ± 0.10	99.5

The values are mean ± se of three observations each.

Over all % reduction with coupling agent >1 mg was 99.6 ± 0.1, n=12

Table III. Linear relationship between NO_2-N mass and absorbance values

NO_2-N (µg in 5 mL volume)	Absorbance (mean ± s.e.)	n
0.05	0.030 ± 0.001	3
0.15	0.090 ± 0.001	3
0.20	0.127 ± 0.003	6
0.40	0.230 ± 0.001	6
0.50	0.300 ± 0.002	6
0.80	0.438 ± 0.003	6
1.00	0.542 ± 0.006	6
1.20	0.607 ± 0.003	3
1.60	0.795 ± 0.003	3

0.05 to1.0 µg N: r = 0.999, b = 0.5345
0.05 to 1.6 µg N: r = 0.998, b = 0.4914 n is number of observations per treatment

Table IV. Linear relationship between NO3 -N mass and absorbance values

NO_3-N µg in 3.3 mL sample	Absorbance values
0.15	0.026 ± 0.001
1.50	0.203 ± 0.001
3.00	0.349 ± 0.002
5.00	0.598 ± 0.003
6.00	0.733 ± 0.002

r = 0.999, b = 0.1189, The values are mean ± se of twelve observations each.

Table V. Estimated nitrite and nitrate in test samples by proposed protocol

Sample	NO_2 -N $\mu g\ g^{-1}$	NO_3 -N $\mu g\ g^{-1}$
Sheep fecal pellets	0.88 ± 0.07	2398 ± 51
Top soil	0.054 ± 0.002	7.99 ± 0.07
Kohlrabi		
Leaves	0.145 ± 0.003	275± 13
Stems	0.523 ± 0.011	447± 20
Common Kashmiri Saag	0.23 ± 0.03[a]	168 ± 7[a]
leaves	0.62 ±0.01[b]	238± 8[b]

The values are mean ± se of six observations each; [a] reduced nitrite and nitrate contents are observed due to some unidentified interfering agent with significant improvement in the contents[b] seen with charcoal treatment @ 10 mg mL^{-1} extract before centrifugation and filtration ($P<0.01$).

RESULTS AND DISCUSSION

Nitrate recovery with charcoal treatments

The adsorption of nitrite and nitrate to activated charcoal, observed in presence of plain water, is remarkably reduced in presence of high electrolyte load and to the extent of 95.5 % in presence of sodium acetate[7]. This provided rationale to use suspension of activated charcoal in acetate buffer to enable selective separation of nitrate from the sample containing nitrite and nitrate in the same sample.

As apparent from Table I, the charcoal suspension in acetate buffer did not affect nitrate recovery. Absorbance values in aliquots containing 0.15, 1.5 and 5 µg of NO_3 –N were not significantly different in charcoal treated samples in acetate buffer compared to the samples treated with acetate buffer alone ($P>0.1$, Table 1). Overall per cent recovery at different masses of NO_3 –N was found to be 101. 5 ± 1.7 (n = 18) in presence of charcoal.

At 25 µg NO_3 –N mass, recovered mass following treatment with charcoal in acetate buffer was found to be 25.4 ± 0.4 µg compared to 20.2 ± 0.2 µg with charcoal in water indicating per cent recovery, correspondingly, as 101.5 ± 1.6 and 80.6 ± 0.7. The absorbance value with charcoal in acetate, 0.524 ± 0.008, was significantly higher than the value, 0.448 ± 0.004, observed in presence of charcoal in water ($P<0.01$, n = 6). This indicated that presence of acetate significantly improves nitrate recovery by preventing adsorption of nitrate to activated charcoal as has been demonstrated previously as well[7].

Filtrates from samples, containing 50 µg NO_2 –N and 7.5 µg NO_3 –N, were colorless suggesting that the charcoal treatment removed all the nitrite from the sample as azo-dye and as any diazotized sulfanilamide. Activated charcoal has remarkable efficiency in adsorption of sulfanilamide[6]. The absorbance values for nitrate in presence of nitrite, 0.212 ± 0.002 did not differ from standard nitrate in absence of nitrite, 0.212 ± 0.003 (n=6, each; $P>0.1$) and NO_3 –N recovered was estimated to be 7.5 ± 0.1 µg showing 100 ± 1 % recovery. This indicated high nitrite did not interfere with recovery of nitrate.

Nitrate recovery following prolonged adsorption

NO_2 –N determination was linear over the test range with absorbance values at 0.2, 0.5 and 1.0 µg, respectively, 0.133 ± 0.002, 0.305 ± 0.003 and 0.556 ± 0.003 (r = 0.999, b = 0.526). NO_3 –N recoveries with 5, 10 and 20 µg were found to be, respectively, 92 ± 2, 86 ± 2, and 85 ± 1 with over all per cent recovery of 88 ± 1 (n= 18). This indicated that even with very prolonged treatment with activated charcoal in acetate buffer of about 20 hours, loss in nitrate estimation was hardly about 12 per cent.

Capacity of charcoal suspension to remove azo-dye and coupling agent

Filtrates obtained from original reaction mixtures containing NO_2 –N as 50, 75, 100 and 125 µg were colorless implying complete removal of azo-dye by the charcoal treatment. The filtrates failed to develop color when added sulfanilamide, NEDA and HCl for nitrite determination. This indicated that the filtrate is free of nitrite, and the anion has been completely consumed in diazotization and coupling reaction, and having got adsorbed to the charcoal as azo-dye. Stoichiometrically 1 mg of coupling agent, used in the present setting, is sufficient for 50 µg of NO_2 –N. The study indicated that the charcoal treatment is capable of removing up to at least 125 µg NO¬2 –N from the sample.

Optimized technique for determination of coupling agent indicated that the diazo reagent was capable of detecting 0.5 through 10µg of coupling agent in 1 mL sample with perfect linearity (r=0.999, b=0.0449, n = 6 each). Absorbance values at 0.5, 1, 3, 5, 10, 12 and 18 µg of coupling agent were respectively found as 0.033 ± 0.003, 0.053 ± 0.003, 0.153 ± 0.003, 0.247 ± 0.003, 0.457 ± 0.003, 0.513 ± 0.003 and 0.670 ± 0.006. Coupling agent was not detectable with initial mass of 1 mg used for nitrite determination in present setting

while recovered masses in 1 mL filtrate at 2, 3, 4 and 5 mg initial levels in 5 mL volume did not exceed 5µg mL^{-1} indicating an overall per cent reduction of 99.6 ± 0.1 (Table II). Presence of coupling agent 1 through 100 µg mL^{-1} with various NO_3-N masses of 3, 5 and 6 µg at elevated temperature with acid reduction did not affect nitrate determinations. Absorbance values in absence of coupling agent at 3 and 6 µg NO_3-N observed, respectively, 0.353 ± 0.003 and 0.732 ± 0.004, were not significantly different from those observed in presence of coupling agent 1 and 10 µg, respectively and correspondingly, 0.350 ± 0.004 & 0.350 ± 0.002, and 0.734 ± 0.002 & 0.724 ± 0.004 ($P>0.1$; n = 5 each). The absorbance values at 0, 10 and 100 µg coupling agent with NO_3-N 5 µg were respectively found as 0.593 ± 0.003, 0.598 ± 0.006 and 0.588 ± 0.005 ($P>0.1$). The study indicated that the charcoal treatment efficiently removed any uncoupled coupling agent, and any mass in the filtrate is unlikely to interfere with nitrate determination under test conditions. In the present setting 1 mg coupling agent has been quite sufficient for determination of nitrite, while 2 mg coupling agent has been sufficient for determination of nitrate in presence of very high acid concentration.

Nitrite and nitrate determination in test samples by proposed protocol

The proposed protocol worked efficiently on test samples. Using randomized pooled data collected during the investigations, the technique for nitrite determination in the present set up has been linear over the test range 0.05 through 1.6 µg NO_2-N ($r = 0.998$, $b = 0.4914$, Table III) with a steeper sensitivity over the range 0.05 through 1 µg NO_2-N in 5 mL volume ($r = 0.999$, $b = 0.5345$). NO_3-N has been linear over 0.15 through 6 µg range at measuring stage in 3.3 mL volume ($r=0.999$, $b=0.1169$, Table IV).

A judicious combination of lead acetate, sodium hydroxide and magnesium chloride as extraction protocol in the present study has been already demonstrated to enable extraction of NOx from different samples ensuring removal of potential interfering agents and providing over 95 per cent mean recovery with nearly 3 per cent accuracy and precision[8]. The coefficients of variation in determinations of NOx based on Griess reaction have ranged from 1.3 – 9.0 % [9, 10]. NO_2-N, as ppm, in soils are normally negligible[11] while NO_3-N range from ca. 0.7 – 13.6 ppm[12, 13]. Nitrogen content in sheep fecal matter is considerably high, ca. 9000 ppm, and nearly double than the levels found in cattle or horse manure[14]. The levels of nitrite and nitrate as nitrogen in test samples during the present investigation (Table V) are well within the documented values for respective samples including those reported from the present laboratory[8]. Interestingly mean NO_3-N content in the sheep fecal pellets (Table V) is not significantly different from the mean values published earlier ($P>0.1$, n= 6 each) while as NO_2-N content was significantly lower ($P<0.01$, n= 6 each) [8]. Similarly, mean NO_3-N concentration in soil as about 8 ppm is comparable to the mean already listed, 9 ppm, for the soil from the adjacent locality[8]. The two leafy vegetables, common Kashmiri saag and kohlrabi, were chosen for the present study as these constitute the most staple diet in Kashmir. Nitrate and nitrite values in the leafy vegetables show characteristic peculiarities. Mean NO_2-N content as ppm in the test leafy vegetables is in the range of 0.145 through 0.62 with content in the leaves of common Kashmiri saag nearly 4.3 times of that present in the leaves of kohlrabi ($P<0.01$) while those of NO_3-N are quite comparable, correspondingly as 238 and 275 ppm ($P>0.1$). Nitrite and nitrate contents in stems of kohlrabi are, respectively, 3.6 and 1.6 times than those in leaves. These findings suggest that the consumption of kohlrabi stems is significantly more important contributor to body NOx load than that of its leaves. NO_3 –N in leafy vegetables in India are reportedly in the range[15] of ca. 7 through 61 with higher content range, 16 through 969 ppm, recorded in spinach[16]. Overall, the levels of the anions in the leafy vegetables are well within the regulatory limits of NOx fixed for the vegetables[17].

CONCLUSIONS

A new method has been standardized to enable sequential and selective determination of nitrite and nitrate by Griess reaction in the same sample containing both anions. The method employs activated charcoal in acetate buffer to facilitate separation of the two anions. The method has been applied to test environmental samples with obvious utilities. The method enables determination of the two anions in the same sample: nitrite is first determined at room temperature under mild acidic conditions followed by removal of azo-dye and reagents by activated charcoal in acetate buffer and then determination of nitrate by acid reduction and coupling at elevated temperature.

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